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Original Research Article

Impacts of Gama Irradiations on the Development of New Mutants in *Stevia rebaudiana* Bertoni

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Abstract: *Stevia rebaudiana* Bertoni is known for its production of sweet glycosides, an alternative to sugar. The cultivation of stevia in Malaysia is hampered because- it produces flowers at an early stage under Malaysia's photoperiod condition, thus leading to poor leaf yield for sweetener extraction. Additionally, there has been low breeding work for suitable cultivars. Thus, the impact of Gama irradiation in developing new *Stevia* mutants was studied. Seeds of MS012 were collected and exposed to gamma irradiation using a gamma cell in UKM, Malaysia to determine the LD_{50}. The seeds were packed into 21 parts; one part served as the control and the others were subjected to varying gamma irradiation grays of 100 to 2000 grays. Irradiated seeds were sown for germination under the red light for 15 days. The LD_{50} was determined by plotting a simple regression graph of plant height against irradiation grays. Results revealed LD50 was 55 grays. The number of germinated seeds differed with respect to doses. Genetic analysis showed four types of chlorophyll mutants were induced: Albina, Chlorina, Xantha and Xantha-Viridis. Frequency and spectrum analysis revealed that Chlorina has the highest occurrence at 33.03%. Two classical novelties: (i) M_1RAAMBO/03, and (ii) M_2 RAAMBO/04 were developed.

Keywords: Gama, irradiation, MS012 Seeds, Cultivation, Photoperiod, Mutants, Stevia.

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Stevia rebaudiana Bertoni, also known as sweet leaf, or sugar leaf, is one of the 950 genera of the family Compositae (Asteraceae) and constitutes about 154 species of herbs and shrubs (El-Mesallamy, *et al.*, 2018). The species is native to Paraguay in Southern America (Gantait *et al.*, 2018), and is one of the only two that produce sweet steviol glycosides (Gunasena *et al.*, 2021).

Koehler, (2015) reported that it was long known to the Guarani Indians of the Paraguayan highlands who called it caá-êhê, meaning sweet herb. The leaves were used either to sweeten maté or as a general sweetening agent.

Stevia is considered a good sugar substitute. Diabetic patients can take herbal powder of stevia available in marketplaces as it lowers the sugar level, giving the sweet taste better than sugar. Stevia produces sweet glycosidic compounds which are non-caloric and the sweet compounds pass through the digestive process of the body without chemically breaking down, thus making stevia a safe substance for consumption for diabetic patients who need to reduce the sugar content of their blood (Rojas *et al.*, 2018).

From most of the previous work, stevia has been reported to have no adverse effect on humans (Latarissa *et al.*, 2020). The leaves could be eaten fresh or dried and they could be boiled in tea to release the sweetener. It has been used for centuries by the Guarani Indians of Paraguay, where the plant originated from, as sweeteners for mate tea (Solanki *et al.*, 2018).

1.2 Mutation breeding

In contrast to conventional breeding where new genetic combinations are produced from pre-

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existing parental genes, nuclear technology causes completely new gene combinations with high mutation frequency. Ionizing irradiation has been the fundamental tool in nuclear technology for developing better cultivars of plants in agricultural practice through mutation induction (Shelake *et al.,* 2019).

Several mutant varieties have been raised in cereals, legumes, vegetables, fruits and ornamental plants (Ricroch, 2021). Genotypic differences are induced in a plant species due to the effects of gamma irradiation grays hence diverse genetic bases are created in plants, especially in cases of risk of extinction or low genetic divergence.

Mutagenesis has been widely applied in the field of crop improvement to generate important plant traits like size, flowering time, fruit ripening and colour, disease resistance and drought tolerance. The number of cultivars derived from mutation induction over the world has increased tremendously (Voss-Fels *et al.,* 2019).

In the development of Quality Protein Maize (QPM), the protein in the endosperm of maize is deficient in two essential amino acids namely, lysine and tryptophan. The opaque 2 mutant genes, along with endosperm and amino acid modifier genes were used for the development of QPM varieties. Results revealed that the QPM has almost twice as much lysine and tryptophan and 30% less leucine compared to normal maize and has been shown to have intense effects on human and animal nutrition, growth and performance. Currently, these QPM varieties are grown on hundreds of thousands of hectares (Wu *et al.,* 2010).

1.3 Classification

The classification of this plant is as shown below:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Compositales

Family: Compositae

Genus: *Stevia*

Species: *rebaudiana*

1.4 Plant Description

Stevia exists as both herbs and shrubs (Jahangir *et al.,* 2020). The plant, under cultivation, can reach up to 1 m or more in height (Libik-Konieczny *et al.,* 2021). It possesses a widespread root system and strong stems producing small, elliptic leaves. The leaves are sessile, alternately arranged in lanceolate to oblanceolate, ovate and serrated above the middle edge. Trichome structures are found on the leaf surface and sometimes on stems (depending on varieties) and are of two different sizes, (Gunasena *et al.,* 2021). The tiny white florets are perfect, borne in small corymbs of 2–6 florets. Corymbs are arranged in loose panicles (Pandey *et al.,* 2021). Gantait and Banerjee, (2018), considered stevia as self-incompatible, insect pollinated and tan seeds as non-fertile. Seeds are contained in elongated achenes, about 3 mm in length. Each achene has an average of 20 persistent pappus bristles.

1.5 Cultivation

Stevia is now cultivated in most parts of the world. Being a sugar substitute and seeing the percentage of diabetes patients in the world, this herb has gained popularity widely. It is commercially cultivated in many parts of Brazil, Paraguay, Uruguay, Central America, Thailand, India and China. In China, stevia is cultivated on large scale. In 2009, extracts from stevia output amounted to about 4,000 tons (Research Report on China's Stevia Extract Industry, 2011). The largest global supply volume of stevia comes from China (Shivani *et al.,* 2019), which is exported to Malaysia, Mexico, the U.S.A., Japan and Hong Kong.

Stevia prefers moist, sandy and loamy soil with high organic matter and adequate drainage. It tolerates a wide range of soil pH. It also prefers partial shade with considerate summer sunshine (Alamgir *et al.,* 2017).

Stevia is grown as a perennial in subtropical regions including the United States but must be grown as an annual in mid to high-latitude regions, where longer days favour leaf yield and stevioside contents (Velazquez, 2021). Propagation of stevia is usually by stem cuttings, which root easily but require high labour inputs. Poor seed germination (10%) possibly due to self-incompatibility is one of the factors limiting large-scale cultivation (Kulus, 2019).

Leaves of stevia are dehydrated in sunshine or heaters and if they are eaten fresh, it gives a bitter taste. Dry leaves are further powdered to be used as a sweetener. Leaves are preferably collected in autumn a transition season between summer and winter when leaves fall naturally. Dead leaves and black leaf spot diseases in stevia are caused by *Septoria* and *Alternaria* species, fungi that live in the soil (Lakshmi and Sivakumar, 2017).

1.6 Uses

In China, Korea, Brazil, Paraguay, Japan and many other countries, stevia and its stevioside extract are used as a tabletop sweetener because they have zero calories, zero carbohydrates and zero fats (Amarakoon, 2021). Being an alternative to sugar, it does not have harmful effects on humans (Latarissa *et al.,* 2020) and can be used in tea, chocolate, and soft drinks. Stevia is added to ice cream and beverages to lower the calorie level and to give a natural taste. Additionally, drinking stevia tea or stevia enhanced-teas helped to reduce a person’s desire for tobacco, alcoholic beverages, as well as the desire for sweets and fatty foods.

Stevia is also used for cavity prevention. In China, for example, stevia is used as an ingredient in certain toothpaste and mouthwash. Stevia and stevioside have been shown to inhibit the growth and reproduction of bacteria that are responsible for tooth decay, thus making them an important ingredient in toothpaste (Estafanos, 2020).

Stevia benefits diabetic patients due to its low calories (Ajami *et al.*, 2020). As herbal medicines, stevia stimulates alertness and counter fatigue, facilitates digestion and gastrointestinal functions, nourishes the liver, pancreas, and spleen, helps the body sustain a feeling of vitality and well-being and is also used for body weight loss (Ahmad *et al.*, 2020). Stevia has been extensively used to control high blood pressure (Ajami, *et al.*, 2020). Recent medical research has shown a promising effect of stevia in treating obesity (Ahmad, *et al.*, 2020).

Other properties of stevia include anti-hypertensive (Ilias *et al.*, 2021), anti-hyperglycemic (Singh *et al.*, 2019) and antioxidant (Khatami *et al.*, 2019).

1.7 STATEMENTS OF THE PROBLEM

(i) Stevia produces flowers at an early stage under short day length Malaysia conditions. When flowers are formed the leaf development is impaired and this is a disadvantage because the leaves are the most needed part of the plant for sweetener extraction.
(ii) Stevia accessions in Malaysia are exotic and do not grow well under local conditions, and there has been little or no breeding work and breeding techniques to develop suitable varieties in the local environment.

1.8 JUSTIFICATION

(i) Stevia helps to control blood glucose levels. Consequently, it has been used as a non-sugar sweetener in food and drinks and as a remedy for diabetes (Ajami *et al.*, 2020).
(ii) New mutants that are insensitive to photoperiod would produce a high leaf yield for sweetener extraction.
(iii) Medical research has shown that stevia has promising effects in treating obesity (Ahmad, *et al.*, 2020), this would help people with obesity problems manage their health.
(iv) Stevia has the potential as a newly emerging crop for Malaysia.

1.9 SIGNIFICANCE OF THE STUDY

New protocols on breeding programs would facilitate raising stevia varieties, enhance better production technology, and increase vegetative yield under local environmental conditions. High leaf yield in new varieties implies a high quantity of extractable sweet stevia glycosides. With promising potential and technical know-how, plans for the stevia industry and business could be drawn and initiated in Malaysia.

Stevia glycosides help to control glucose levels; consequently, they can be used as a non-sugar sweetener for food and drinks and as a remedy for diabetes.

1.10 OBJECTIVES OF THE STUDY

1.10.1 Main objective

The main objective of this research is to study the impact of Gama irradiation in developing new Stevia mutants suitable for cultivation under Malaysia's conditions.

1.10.2 Specific objectives

The specific objectives in the current studies are listed below:

(i) Mutation induction by gamma irradiations to increase genetic variability to develop new cultivars of stevia that fit for proper growth and high yield under local environment, through evaluating M_1 to determine the LD_{50},
(ii) Screening of the chlorophyll mutants in the M_2 seedlings, and characterization of morphological mutations in M_2 seedlings and M_2 plants for selection of new mutants.

2. MATERIALS AND METHODS

2.1 Mutation breeding by gamma irradiation

In this study, efforts were made to raise new genetic variants by exposing MS012 seeds of Stevia to varying grays of gamma irradiation. Studied traits of interest include insensitivity to photoperiod and high vegetative yield with a focus on the leaves.

2.2 First gamma irradiation experiment- Seeds exposed to 100 – 200 grays

The lethal dose (LD_{50}) of gamma irradiation is plant species specific. Seeds of MS012 were collected and exposed to gamma irradiation using gamma cells (220 Excall, Nordean, Canada) at the Universiti Kebangsaan Malaysia (UKM). The seeds were packed into 21 parts; one part served as the control and the others were subjected to varying grays of gamma irradiation ranging from100 to 2000 grays. Irradiated seeds were sowed for germination under the red light for 15 days.

All seedlings died at a very early stage due to high irradiation grays. A lower irradiation dose was used in the next experiment.

2.3 Second gamma irradiation experiment- Seeds exposed to 0 – 100 grays

Seeds MS012 were collected and exposed to gamma irradiation ranging from 0 to 100 grays. Seeds were sown under red light, and the numbers of germinated seeds after 3 weeks along with the respective seedling heights were recorded. The LD_{50} was determined by plotting a simple regression graph of plant height against irradiation grays. Having determined the LD_{50} to be 55 grays (dose) in this experiment, third gamma irradiation was carried out.

2.5 *Third gamma irradiation experiment- Seeds exposed to 45, 55 and 65 grays*

Seeds were exposed to gamma irradiation at 45, 55 and 65 grays. The M_1 seeds were germinated and nurtured in the nursery for 25 days. Seedling heights were measured and another regression curve was plotted. The young seedlings were transferred to the field. The M_1 plants were allowed to develop until they were 10 weeks old and produced seeds (M_2).

2.6 Chlorophyll mutants in the M2seedlings

The use of chlorophyll mutant assay to confirm and evaluate mutants in plants subjected to mutations has been a normal field practice (Miglani *et al.*, 2021).

In this study, matured M_2 seeds were harvested and germinated. At an early stage of the seedlings' development, (1 to 21 days old) seedlings were screened using both genetic chlorophyll mutants expressions and morphological parameters.

Observation for lethal and non-lethal chlorophyll mutations was made from day 1 of the emergence of germinated seeds to day 20. Identification and classification of chlorophyll mutations were carried out according to Miglani *et al.*, (2021) when the young seedlings were 8 to 20 days old. In this study, lethal mutations were Albina and Xantha while non-lethal mutations were Chlorina and Xantha-Viridis.

The characteristics of the mutants are listed below:

Albina: Characteristically white, it is lethal, neither chlorophyll nor carotenoids were formed.

Xantha: Characteristically yellow to yellowish white, it is lethal and has no chlorophyll but carotenoids are present.

Chlorina: characteristically uniformly light green, non-lethal, has chlorophyll and carotenoids.

Xantha-Viridis: initially light/yellow green, non-lethal, because as seedlings grow it turns to green colour (possible heterozygous group).

Non-defined: leaf chlorophyll colour did not match the characteristic patterns of chlorophyll deformities.

To study the genetic phenomenon, segregation frequency and mutagenic effectiveness were calculated according to Miglani *et al.,* (2021). The formula used is as follows:

(i) Segregation frequency: Average number of mutant seedlings per plant/Average number of M_2 seedlings x 100.
(ii) Mutagenic effectiveness: M x100/grays,

Where,

M= chlorophyll mutation frequency for 100 M_2 plants;
Grays= dose of mutagenic radiation in grays.
Chlorophyll mutation frequency= Number of mutants/100 M_2 plants x100.

Statistical analysis was carried out using one-way ANOVA at $p < 0.05$. Photos of gamma cells, mutant seedlings and plants were taken.

2.4 Evaluations of morphological mutations in M2 seedlings and plants

Apart from chlorophyll mutants, other forms of morphological mutations observed in M_2 seedlings were (i) period of flowering, (ii) plant height, (iii) number of branches, (iv) number of leaves, (v) leaf size and (vi) stem girth. Twelve weeks old M_2 plants were evaluated.

3. RESULTS

3.1 Mutation breeding by gamma irradiation

In this experiment, stevia accession MS012 was used for mutation studies. Gamma cell in UKM was utilized for mutation induction by exposing the seeds to different irradiation doses (grays) to determine the LD_{50} needed to induce mutation in stevia. In summary, the LD_{50} was 55 grays and different types of mutations were induced in stevia plants.

3.2 First gamma irradiation experiment

In this experiment, seeds irradiated with different gamma doses (grays) show variation in the number of germination (Figure 3.1). However, the germinated seeds failed to grow; they remain stunted and subsequently die perhaps due to high gamma irradiation effects (100 – 2000 grays).

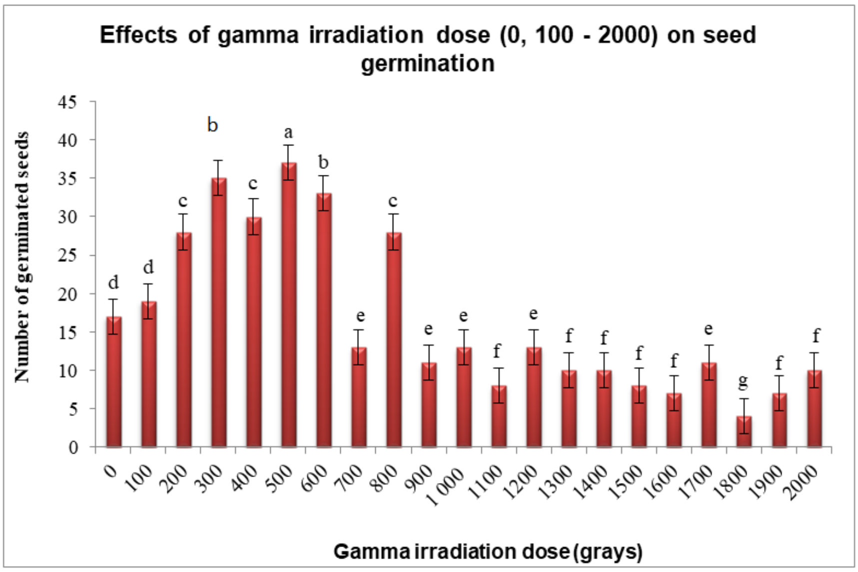


Figure 3.1: Effects of gamma irradiation dose (0, 100 – 2000) on seed germination

Several germinated seeds treated with gamma rays are shown. Different alphabets denote significant difference ($p < 0.05$)

The average number of germinated seeds ranged from 4 to 37 across varying gamma irradiation grays (0, 100 – 2000) at $p < 0.05$. Although there are germinated seeds across different treatments, their heights could not be taken, as they all remain stunted and subsequently withered.

3.3 Second gamma irradiation experiment

In this second round of the experiment, a lower dose of irradiation was used (ranging from 0 to 100). The seeds germinated and the epicotyls elongated in all gamma irradiation treatments, except for the 90 and 100 grays, which may be due to the higher dose effects of the irradiations (Figure 3.2). Figure 3.3 showed stages involved in mutation induction from irradiated seeds to M_1 plants.

The heights of seedlings in respect of varying irradiation grays indicated higher values with a smaller dose than the higher dose of gamma irradiations (Figure 3.2). For example with smaller doses of 0, 10, 20, and 30 grays, the respective height values of seedlings were 6.15, 4.90, 4.30 and 4.15cm, while for higher doses like 60, 70 and 80 gray their respective height values were 3.25, 2.80 and 2.70 cm ($p < 0.05$). No heights were taken for 90 and 100 grays because the germinated seeds did not grow and later died.

The LD_{50} can be determined by measuring seedling heights and the data analyzed using a regression curve. The regression curve in Figure 3.2 showed the illustration of the results. The LD_{50} was found to be 55 grays as shown on the graph.

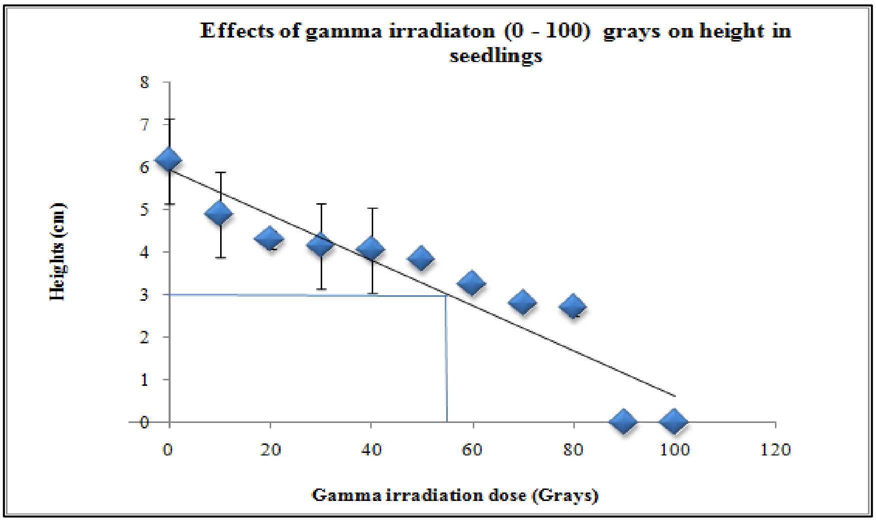


Figure 3.2: Simple linear regression curve on heights of seedlings due to gamma irradiation doses (0 -100 grays). The appropriate LD_{50} for stevia is 55 grays of gamma irradiations

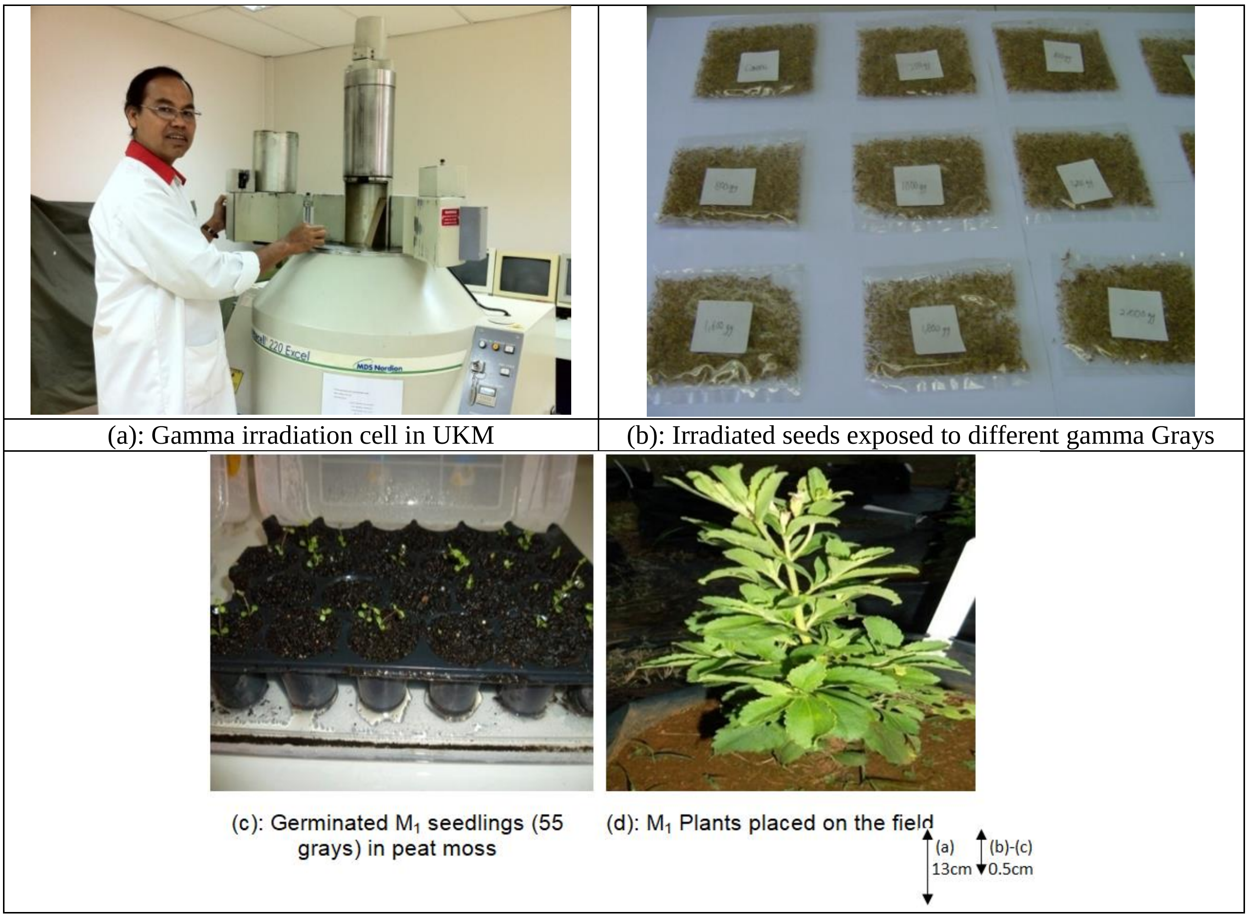


Figure 3.3: Stages involved in mutation induction from seed to M_1 plants

3.4 Third gamma irradiation experiment

The third gamma irradiation experiment was conducted by exposing seeds to three different irradiation doses: 45, 55 and 65 grays. The second regression curve in Figure 3.4 illustrated the influence of gamma irradiation doses 45, 55 and 65 on seedling heights in the raised M_1 population ($p < 0.05$). The R2 value of 0.996 was obtained from the linear regression curve. This indicated that more than 90% of the variable i.e. plant heights of seedlings were influenced by the variable: gamma irradiation doses applied for treatments. Overall this shows a high correlation between the two variables and thus the accuracy of the result obtained.

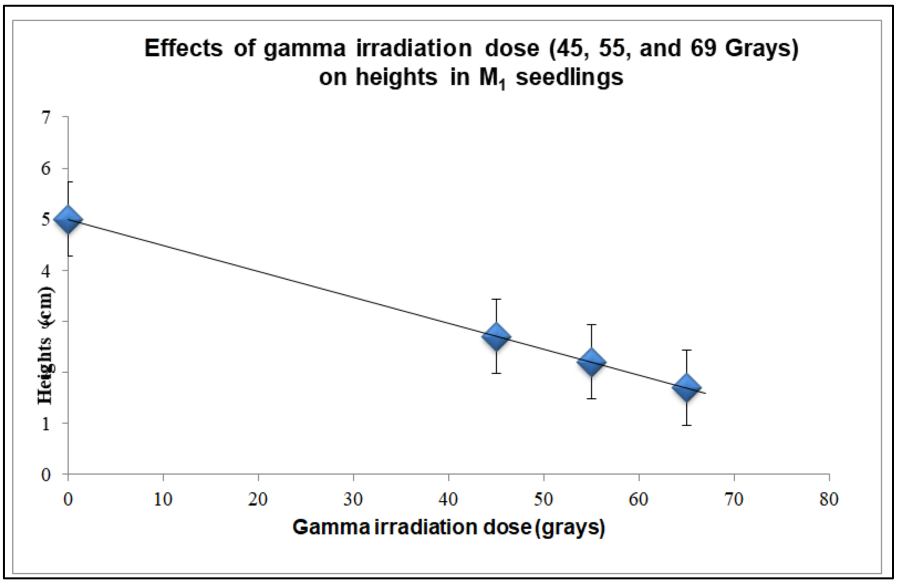


Figure 3.4: Effects of gamma irradiation dose 45, 55 and 65 on heights in M_1 seedlings

3.5 Chlorophyll mutants in the M_2 seedlings

3.5.1 Chlorophyll mutant markers

The chlorophyll mutant markers were used to investigate the effectiveness of the gamma irradiation dosage to induce mutation in this crop. 2,100 M_2 seeds were sown, and 110 germinated, out of which 78.62% were mutants. Four types of chlorophyll mutants were observed across the range of gamma irradiation. They are Albina, Chlorina, Xantha and Xantha-Viridis (Figure 3.5), the description of the mutants is shown in Table 3.1.

The overall frequency and spectrum of chlorophyll mutation in M_2 generation are shown in Table 3.2 and it revealed that Chlorina has the highest frequency of occurrence with 33.03 % over Xantha which was 20.48, Albina with 15.7, and Xantha-Viridis with 9.41 %. All the four mutations were expressed owing to every gamma irradiation grays, except for 60 and 70 grays where Albina and Xantha were absent.

Figure 3.5: Spectrum of chlorophyll mutants due to varying gamma Irradiations grays

Table 3.1: Description of the chlorophyll mutant characters observed in the M_2 seedlings

Types of chlorophyll mutations	**Description of Mutant Character**
Albina	Leaf almost white, absence of chlorophyll and carotenoids = LETHAL
Chlorina	Leaf uniformly dull green, presence of chlorophyll and deficiency of carotenoids = VIABLE
Xantha	Leaf is yellow/pale, absence of chlorophyll and carotenoids = LETHAL
Xantha-viridis	Leaf initially yellow/pale, later turned green presence of chlorophyll and carotenoids = VIABLE

Three main mutations were recorded in the M2 population of stevia, they are chlorophyll (Table 3.1), and leaf and stem colour mutations (Table 3.3). The chlorophyll mutants were made up of both lethal and viable types; the Albina and Xantha were the lethal types, while the Chlorina and Xantha-Viridis are the viable types.

Table 3.2: **Frequency and spectrum of chlorophyll mutants in seedlings of M_2 generation**

Gamma irradiation dose (grays)	Number of M_2 seeds sown	Number of seedlings raised	Relative frequency (%) of chlorophyll spectrum				Percentage (%) of mutants
			Albina	Chlorina	Xantha	Xantha-Viridis	
40	300	12	16.6	16.6	50	8.3	91.5
45	300	12	8.3	25	50	16.6	99.9
50	300	18	11.1	11.1	38.9	11.1	72.2
55	300	21	4.8	14.2	52.4	14.2	85.6
60	300	11	0.0	27.3	9.1	18.2	54.6
65	300	13	7.7	23.1	30.8	15.4	77
70	300	23	17.4	26.1	0.0	26.1	69.6
TOTAL	2,100	110	Relative Mean Value (%)				
			15.7	33.03	20.48	9.41	78.62

With the gamma irradiation dose of 40grays the frequency of mutation (%) are: Albina 16.6, Chlorina 16.6, Xantha 50.0, and Xantha-Viridis 8.3, with the order of mutation Xantha>Albino>Chlorina>Xantha-Viridis. The frequency of mutation with an irradiation dose of 45 grays showed: Albina 8.3, Chlorina 25, Xantha 50.0, and Xantha-Viridis 16.6, thus the order is Xantha>Chlorina>Xantha-Viridis>Albina. The gamma irradiation dose of 50 grays induced the following mutation frequency: Albina 11.1, Chlorina 11.1, Xantha 38.9, and Xantha-Viridis 11.1, the order of frequency is Xantha> Albino= Chlorina= Xantha-Viridis. Furthermore, the 55 grays of gamma irradiation dose caused the following mutation frequency: Albina 4.8, Chlorina 14.2, Xantha 52.4, and Xantha-Viridis 14.2, and they occur in the order of Xantha>Chlorina= Xantha-Viridis> Albina. The 60 gray dose of gamma irradiation induced mutation frequency: Chlorina 27.3, Xantha 9.1, and Xantha-Viridis 18.2, with the order of frequency, follows the trend of Chlorina> Xantha-Viridis> Xantha. With the 65grays, the mutation frequency was: Albina 7.7, Chlorina 23.1, Xantha 30.8, and Xantha-Viridis 15.4, and the order is in the trend of Xantha> Chlorina> Xantha-Viridis>Albino. The highest dose of 70 grays induced mutation frequency: Albina 17.4, Chlorina 26.1 and Xantha-Viridis 26.1, and it follows the order of Chlorina=Xantha-Viridis>Albina.

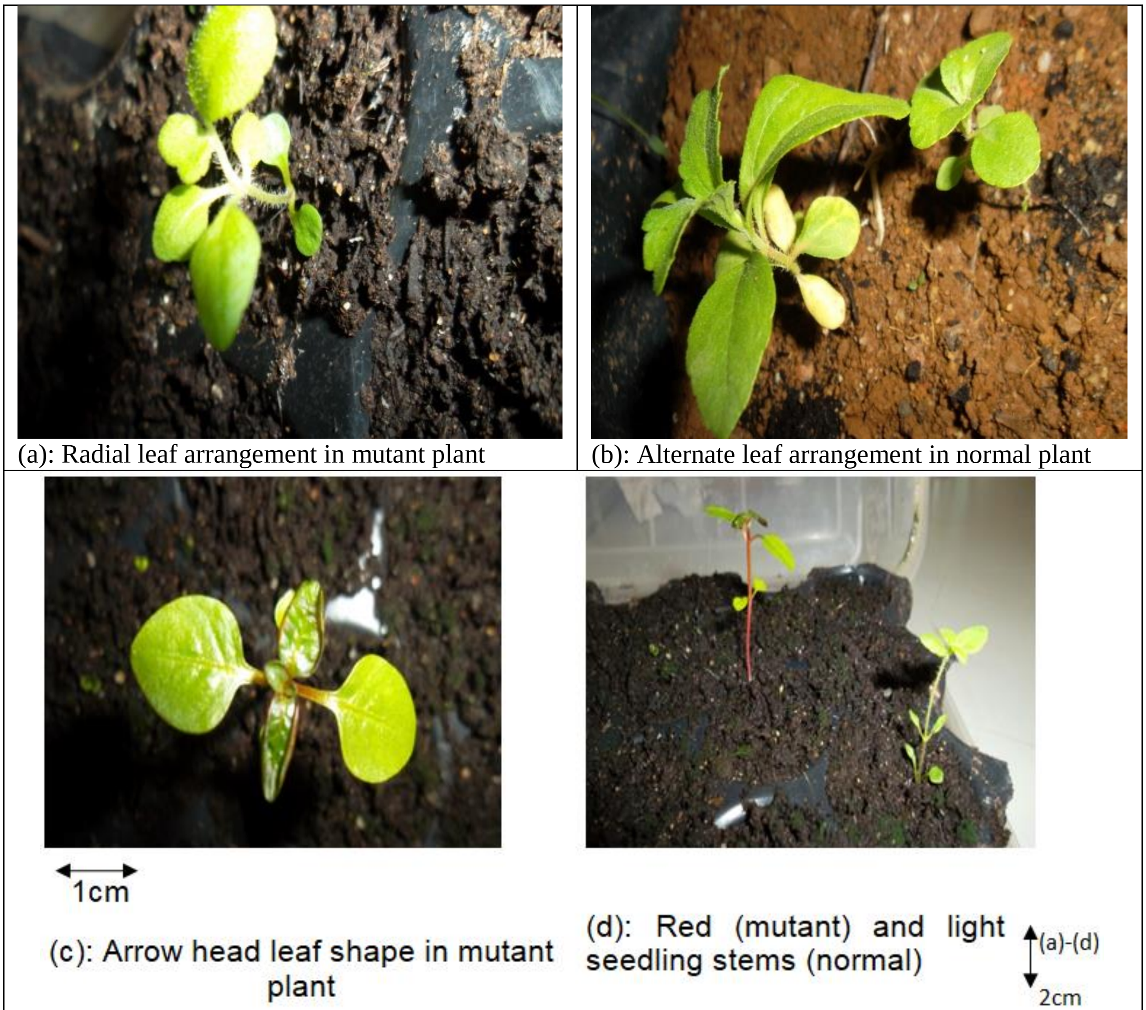
3.5.1.1 Evaluations on morphological mutations in M2 seedlings

Apart from chlorophyll mutants, other forms of morphological mutations in M_2 seedlings were investigated which are the leaf arrangement, shape and stem colour. Qualitative evaluations made on observed mutations in the morphology of young seedlings of M_2 population are stated in Table 3.3. The essence of both chlorophyll and morphological mutants evaluation is to confirm the efficacy of LD_{50} (55 grays) in inducing mutations.

Table 3.3: Morphological evaluations on mutant M_2 seedlings

Observed mutant traits	Description of mutant character
Leaf mutation	
Leaf arrangement	Radial and cluster leaf arrangement observed.
Leaf shape	Lanceolate, arrowhead, narrow & broad leaf shape.
Stem colour mutation	Red colour stems observed

Radial and cluster leaf arrangement mutant types were found as opposed to the normal alternate leaf arrangement type in the control plants (Figure 3.6 (a) and (b)). Leaf shape also differs in mutant plants where they were lanceolate, arrowhead, narrow, and broad leaf shapes (Figure 3.6 (c)) as compared to the ovate shape of leaves in the normal untreated plants. Red stem colour was also observed in the mutant population, which is a deviation from the normal green stem colour in this crop as shown in Figure 3.6 (d). Figure 3.7 showed the M_2 plants of stevia on the field.



(a): Radial leaf arrangement in mutant plant
(b): Alternate leaf arrangement in normal plant
(c): Arrow head leaf shape in mutant plant
(d): Red (mutant) and light seedling stems (normal)

Figure 3.6: **Varying morphological mutations in the M_2 population of stevia**

3.5.1.2 Morphological character evaluations in M_2 plants

M_2 plants were evaluated on these morphological characters: the period of flowering, plant height, number of branches, number of leaves, size of leaves, number of corymbs and stem girth. The results showed that different doses of gamma had varying influences on the morphological characters (Figure 3.8) at $p< 0.05$. All the plants produced flowers at an early stage indicating that they are still subjective to the influence of the Malaysian environmental photoperiod (Figure 3.8 (a).

(a): M_2 plants first row
(b): M_2 plants second r(

Figure 3.7: M2 plants of stevia on the field

The dose of 55 grays influenced the highest value in plant height (44cm) (Figure 3.8 (b)), while the least value (32cm) was influenced due to 70 grays. Furthermore, 45 grays had the highest value (33) on the number of branches (Figure 3.8 (c)), the least value (7) was recorded due to 50 grays. The number of leaves counted ranged from 75 to 106 (Figure 3.8 (d)). Moreover, the large size of the leaves was influenced by 70 grays (20.22 cm), while the least value (9.4 cm) was recorded from 60 gray effects (Figure 3.8 (e)). Most of the individual plants had a high number of flowers ranging from 51 to 205 (Figure 3.8 (f)). The stem girth value was highest (2.47) owing to 55 grays (Figure 3.8 (g)), while the least value (1.40) was recorded with the effects due to 70 grays.

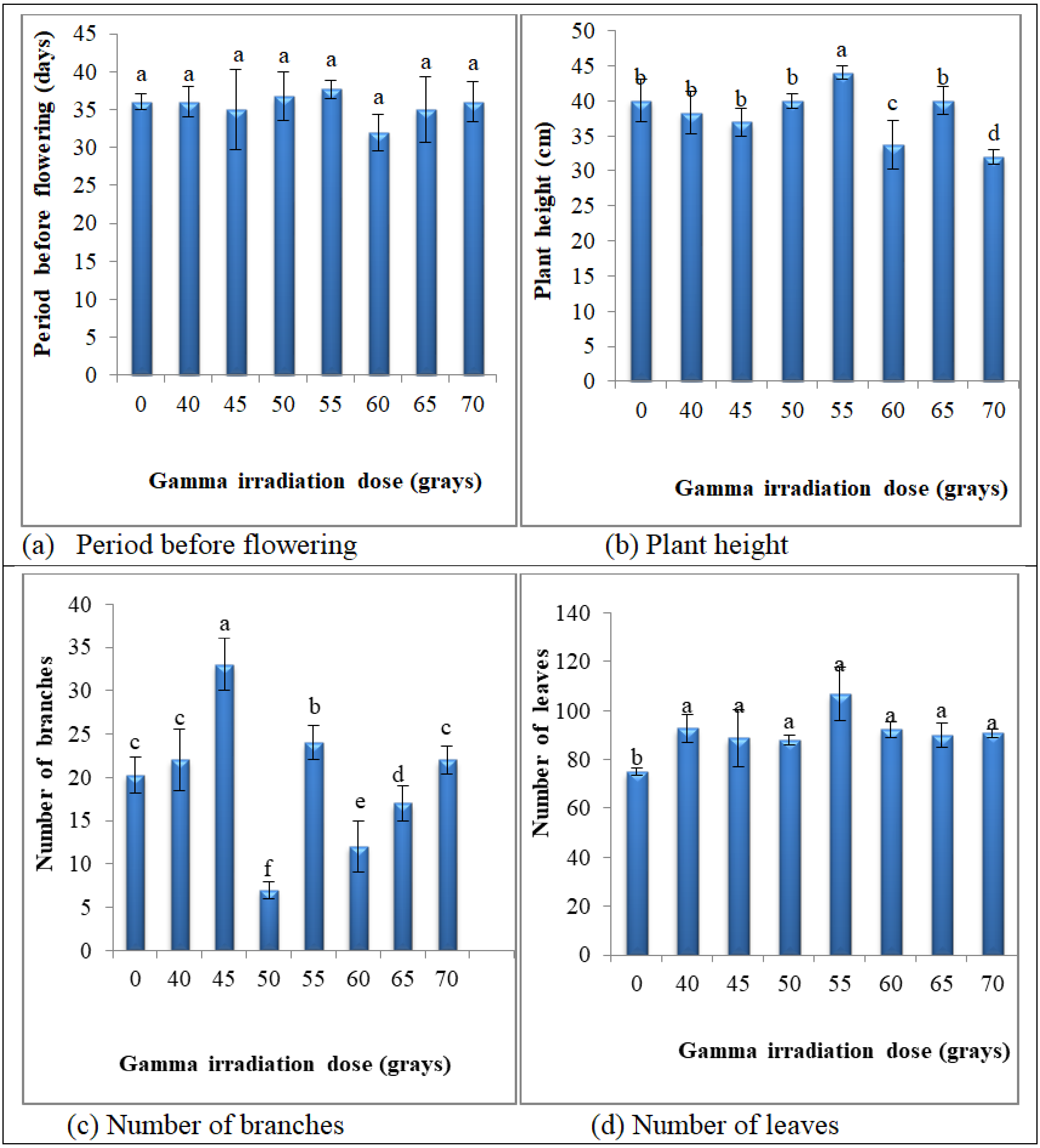
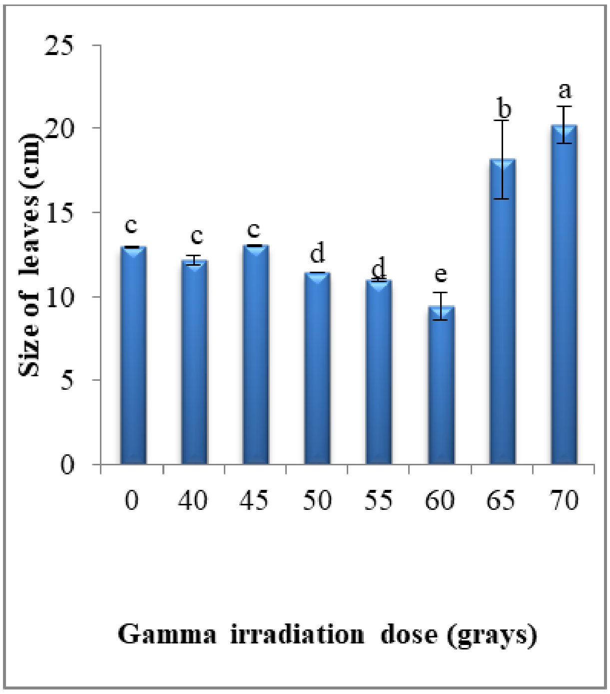
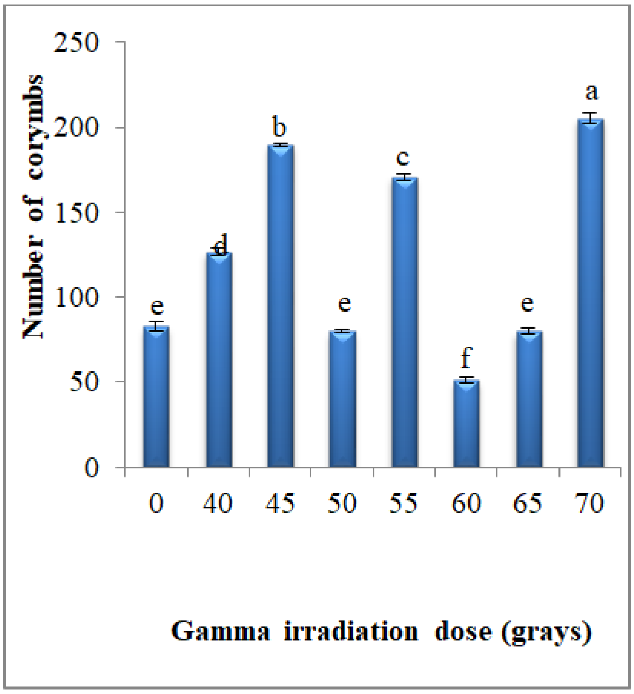


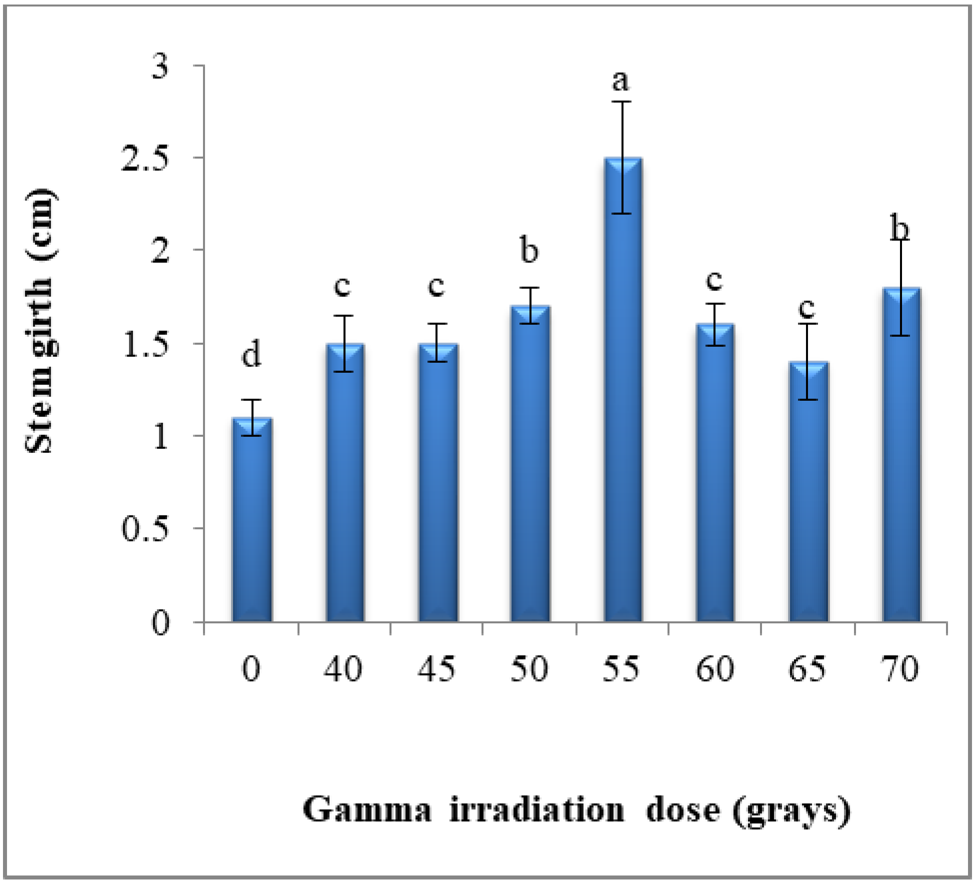
Figure 3.8: **Morphological characters in M2 generation of stevia. Period of flowering, plant height, number of branches, number of leaves, size of leaves, number of corymbs and stem girth are shown. Different alphabets denote significant difference (*p* < 0.05)**



(e) Size of leaves



(f) Number of corymbs



(g) Stem girth

Figure 3.8: **Morphological characters in M_2 generation of stevia. Period of flowering, plant height, number of branches, number of leaves, size of leaves, number of corymbs and stem girth are shown. Different alphabets denote significant difference (*p* < 0.05)**

Two of the most important traits of interest: delay in the period of flowering and high yield in the number of leaves, statistically showed no significant difference ($p > 0.05$). However, the plant height, number of branches, and stem girth differed significantly ($p < 0.05$).

Almost all the plants, irrespective of the gamma irradiation gray produced flowers at an early stage. Some remain dwarf and poorly developed, variations were observed across the plants on evaluated characters due to the effects of different gamma irradiation doses.

The fact that these plants produced flowers at an early stage is an indication that they are still subject to influence by the environmental photoperiod, with stevia being a short-day plant. Raising plants that are insensitive to photoperiod may constitute a breakthrough in this crop. Although the chance to unravel plants of these desired traits, low flowers and high leaf yield, may be low, continuous propagation through breeding and selection, may eventually lead to unknotting the plants of interest.

DISCUSSION

The frequency of mutants (based on genetic and morphological characteristics) confirmed the effectiveness of the gamma irradiation dosage (LD_{50}) to induce mutation in stevia. The M_2 plants produced flowers at an early stage and the vegetative yield is poor. The mutant plants are still subjective to the Malaysian photoperiod. Thus, none of the M_2 plants could be selected for propagation. However, there is a need for continuous crossings and selections until the ideal genotypes are developed. The new mutants constitute rich sources of genetic materials for future breeding work in *Stevia rebaudiana* Bertoni to unravel the germplasm with desired traits suitable for cultivation under Malaysian conditions. The basic tool in nuclear technology for agricultural crop improvement is the utilization of ionizing irradiation. This approach to crop improvement and development is quite different from conventional breeding, where new genetic combinations are developed from existing parental genes. In this case, completely new gene combinations are formed with high mutation frequency (Ahmad *et al.*, 2019).

Due to the effects of gamma irradiation, several physiological changes have been induced in crops. Gamma irradiation potentials have been employed for use in agriculture, industries, and medicines. However, its potential exploitation in agricultural practice is limited due to a lack of information and awareness on optimal doses of irradiations (LD50) which differ from one crop to another and are specific according to species. The radiation that mediates morphological, structural and functional changes in plants is dependent on the intensity and duration of the gamma irradiation (Singh *et al.*, 2020).

According to Parchin *et al.*, (2019), gamma irradiation has also shown a negative effect on plants. The root growth and shoot elongation were considerably reduced in irradiated common and mung beans in comparison to the non-irradiated checks. The LD_{50} that prevented shoot and root elongation varied among species and ranged from genotype to genotype within species. The Kabuli types of mung bean were more sensitive than the desi ones. A higher dose of gamma irradiation of 30 and 35 krad resulted in abnormalities in wheat varieties (Din *et al.*, 2003). For example, a tiller has two ears attached and/or the prevalence of sterile ears.

In contrast, mutagenesis induces many useful traits in plants, including plant size, flowering time and fruit ripening, fruit colour, self-compatibility, self-thinning, and resistance to pathogens. The number of new cultivars raised due to mutations increases constantly (Al-Taweel *et al.*, 2021).

CONCLUSION

Gamma irradiation is highly effective in mutation induction in stevia. The influence of gamma irradiation on the frequency of occurrence and chlorophyll mutation induction varies according to dose. The overall frequency and spectrum of chlorophyll mutation in M_2 generation showed Chlorina has the highest frequency of occurrence with 33.03% over Xantha (20.48%), followed by Albina (15.7%) and Xantha-Viridis (9.41%). The frequency of occurrence in different seedling genotypes could be due to the difference in the location of genes to the chromosomal centromere. Studying the frequency and spectrum of chlorophyll mutation assay assisted in identifying the effective doses that could induce mutations in stevia. The LD_{50} was 55 grays. New mutants with varying mutations in parameters such as leaf arrangement, leaf shape, leaf colour, leaf size, number of leaves, stem colour, plant heights and number of corymbs, were raised. Two mutants were selected as new species under Malaysia's environmental conditions, and they are the M_1 mutant variety, RAAMBO/03 and the M_2 mutant variety, RAAMBO/04. The mutagenesis approach was efficient in the development of new varieties for consumption and/or for a future breeding program.

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